Amendments to the Specification

Please replace the paragraph and its header beginning at page 1, line 2, with the following amended paragraph.

~~RELATED APPLICATIONS~~ CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a U.S. national counterpart application, filed under 35 U.S.C. § 371, of international application serial No. PCT/US2005/006638 filed February 28, 2005, which claims the benefit of ~~priority under 35 U.S.C. §119(e) to~~ US Provisional Application Serial No. 60/549,306, filed March 2, 2004, and 60/604,846, filed August 26, 2004, each ~~the disclosures~~ of which ~~are~~ is incorporated herein by reference.

Please replace the paragraph at page 2, line 10, with the following amended paragraph.

One aspect of the present disclosure relates to compositions and a method of reducing the virulence of pathogenic bacteria by preventing the expression of functional hydrogenase activity in the ~~bacterial~~ bacteria.

Please replace the paragraph at page 2, line 14, with the following amended paragraph.

One embodiment of the present disclosure is directed to an isolated bacterium that has been modified to be hydrogenase deficient relative to wild type strains. More particularly, the modified bacterial strain has a greater than 50% reduction in hydrogenase activity relative to a wild type stain. More particularly, in one embodiment the modified strain is incapable of expressing a functional NiFe hydrogenase protein. In accordance with one embodiment the bacterial strain is an enteric pathogen selected from the group consisting of *Salmonella, E. Coli, Shigella, and Campylobacter (*including for example, *Salmonella typhimurium, Salmonella typhi, E. Coli 0157, Shigella flexneri, ~~Shingella~~ Shigella sonnei, and Campylobacter jejuni*), wherein the strin comprises a mutation to each of the NiFe hydrogenase genes present in the genome of the strain, such that the mutations prevent the expression of a functional NiFe hydrogenase protein.

Please replace the paragraph at page 3, line 1, with the following amended paragraph.

The present disclosure is also directed to the use of compositions comprising hydrogenase deficient bacteria to treat or prevent enteric bacterial pathogenic infections. In one embodiment a method of inducing an immune response in a mammal against a pathogenic bacterium is provided, wherein the immune response protects the animal from, or reduces the severity of, future infections of that pathogen. The method comprises the step of administering to said mammal a composition comprising live bacterium, wherein the bacterium has been modified to prevent the expression of a functional NiFe hydrogenase protein. In one embodiment the bacterium is selected from the group consisting of *Salmonella typhimurium, Salmonella typhi, E. Coli 0157, Shigella flexneri, ~~Shingella~~ Shigella sonnei, and Campylobacter jejuni*, and the composition is administered orally.

Please replace the paragraph at page 7, line 4, with the following amended paragraph.

In accordance with one embodiment of the present disclosure, a pathogenic bacterial cell is provided wherein the NiFe hydrogenase genes of the bacteria have been modified my mutation to prevent the bacteria from expressing a functional NiFe hydrogenase enzyme. In accordance with one embodiment the mutation introduces one or more stop codons into the reading frame of the NiFe hydrogenase gene. Alternatively, the mutation may introduce a frameshift that allows for the generation of a protein that is devoid of, or has very poor, hydrogenase activity. In another embodiment the mutation comprises a deletion of at least a portion of the gene, rendering the gene incapable of expressing a functional NiFe hydrogenase protein. Since several NiFe hydrogenase genes are expressed from operons that encode several gene ~~product~~ products, it is advantageous in these situation to select a mutation strategy that minimizes the impact on downstream gene expression.

Please replace the paragraph at page 9, line 19, with the following amended paragraph.

In accordance with one embodiment a composition comprising two or more pathogenic strains selected from the group consisting of *H. Pylori, H. Hepaticus, Salmonella typhimurium, Salmonella typhi, E. Coli 0157, Shigella flexneri, ~~Shingella~~ Shigella sonnei,* and *Campylobacter*

jejuni, wherein each of the NiFe hydrogenase genes of the respective pathogenic strains has been mutated, wherein the mutations prevent expression of a functional NiFe hydrogenase protein.

Please replace the paragraph at page 9, line 25, with the following amended paragraph.

In accordance with one embodiment of the present disclosure, a method of inducing an immune response in a mammal against a pathogenic bacterium is provided, wherein the immune response protects the animal from, or reduces the severity of, future infections by that pathogen. The method comprises the step of administering to said mammal a composition comprising live bacterium, wherein the bacterium has been modified to prevent the expression of a functional NiFe hydrogenase protein. In one embodiment the bacterium is selected from the group consisting of *Salmonella, E. Coli, Shigella,* and *Campylobacter*. In one embodiment, the bacteria is selected from the group consisting of *Salmonella typhimurium, Salmonella typhi, E. Coli 0157, Shigella flexneri, ~~Shingnella~~ Shigella sonnei,* and *Campylobacter jejuni*, and the composition is administered orally. In one embodiment the immune response is directed against a pathogenic bacterium selected from the group consisting of *Salmonella typhimurium, Salmonella typhi* and *Campylobacter jejuni.*

Please replace the paragraph at page 10, line 5, with the following amended paragraph.

In one embodiment the method of inducing an anti-pathogen immune response in a mammal comprises the step of administering to the mammal, including humans, a composition comprising live bacteria, wherein the bacteria have been modified to prevent the expression of a functional hydrogenase protein. More particularly, in one embodiment each of the NiFe hydrogenase genes of the wild type pathogen have been mutated to prevent expression of each of the NiFe hydrogenase genes present in the genome of the bacterium. In one embodiment the mutation comprises the deletion of the entire gene, or a portion thereof, of the large and/or small subunit of each hydrogenase gene. In one embodiment the modified bacterium is selected from the group consisting of *Salmonella typhimurium, Salmonella typhi, E. Coli 0157, Shigella flexneri, ~~Shingella~~ Shigella sonnei,* and *Campylobacter jejuni*. In [[on]] <u>one</u> embodiment the

modified bacterium is selected ~~form~~ from the group consisting of *Salmonella typhimurium* and *Salmonella typhi,* and in one embodiment the modified bacterium is *Salmonella typhimurium.*

Please replace the paragraph at page 14, line 11, with the following amended paragraph.

That hydrogen present in animals (as a consequence of normal colonic flora metabolism) is an energy yielding substrate for maintenance of a pathogenic bacterium may be extended to a number of (mostly enteric) pathogens. Based on publicly available complete genome sequence annotations (such as the Institute for Genomic Research, the Sanger Institute, or the National Center for Biotechnology Information) a number of human pathogens have genes encoding all the components required for gleaning energy from H_2 respiration. This would include the structural genes for a membrane bound hydrogenase and for shuttling of those electronics to quinone-binding or heme b binding proteins, as well as the accessory proteins for the NiFe hydrogenase enzymes' maturation. These bacteria include *Salmonella entericaserovars Typhi* and *Typhimurium, E. Coli* 0157, *Shigella (flexneri flexneri and sonnei)* and *Campylobacter jejuni.* For some of the enterics, H2 oxidation via hydrogenases has been measured (albeit anaerobically).

Please replace the paragraph at page 15, line 4, with the following amended paragraph.

Based on available annotated gene sequence information the enteric pathogen Salmonella, like other enteric bacteria, contains three putative membrane-associate H_2 using hydrogenase enzymes. These enzymes split molecular H_2, releasing low potential electrons that are used to reduce (quinone or heme-containing) components of the respiratory chain. Here we show that each of the three distinct membrane associated hydrogenases of Salmonella enterica serovar Typhimurium are coupled to a respiratory pathway using oxygen as the terminal electron acceptor. Cells grown in a blood-based medium expressed 4 times the amount of hydrogenase (H_2 oxidation) activity than did cells grown on Luria Broth medium. Cells suspended in phosphate buffered saline consumed two moles of H_2 per mole of O_2 used in the H_2/O_2 respiratory pathway, and the activity was inhibited by the respiration inhibitor cyanide. Molecular hydrogen levels are averaging over 40 microM were measured in organs (i.e. liver and

spleen) of live mice, and levels within the intestinal tract (the presumed origin of the gas), were 4 times greater than this. The half-saturation affinity of Salmonella typhimurium for [[H2]] $\underline{H_2}$ is only 2.1 microM, so it is expected that (H_2-utilizing) hydrogenase enzymes are saturated with the reducing substrate in vivo. All 3 hydrogenase enzymes contribute to virulence of the bacterium in a typhoid fever-mouse model, based on results from mutant strains in each of the 3 genes. The introduced mutations are non-polar, and growth of the mutant strains was like that of the parent strain. The combined removal of all 3 hydrogenases resulted in a strain that is avirulent and (in contrast to the parent strain) one that is unable to invade liver or spleen tissue. Introduction of one of the hydrogenase genes into the triple mutant strain on a low copy number plasmid resulted in a strain that was both able to oxidize H2 oxidation and able to cause morbidity in mice within 11 days of inoculation; therefore the avirulent phenotype of the triple mutant is not due to an unknown spurious mutation. We conclude H2 utilization in a respiratory fashion is required for energy production to ~~permit~~ permit Salmonella growth and subsequence virulence during infection.

Please replace the paragraph at page 16, line 12, with the following amended paragraph.

The conditions for obtaining hydrogenase activity involved growing cells on a blood-containing medium (Olczak, et al., (2002) J. Bacteriol. 184:3186-3193), in a microaerobic H_2 containing atmosphere (see Table 1 legend). *S. Typhimurium* cells grown one day on the BA plates were suspended in a phosphate-buffered saline, and 8 ml samples at cell concentrations of 8×10^8 cells per ml were assayed for H_2 and O_2 uptake activities simultaneously. This was accomplished on the same sample in a stirred and sealed amperometric dual-electrode chamber (Merberg, et al., (1986) Bacteriol. 156:1236-1242). Hydrogen and oxygen were added as needed from gas-saturated solution of phosphate buffered saline. H2 uptake rates were linear until the substrate reached levels of about 3 to about 5 μM. For methylene blue dependent rates, the chamber lacked oxygen but contained MB at 200 μM, and the cells were permeabilized with ~~TritonX~~ TRITONX-100 before assay. Cell numbers were determined by performing dilutions and plate counts on MacConkey medium. For determining affinity of whole cells for H_2, the H_2/O_2 uptake assay was performed in a series of limiting H_2 levels between 1.2 and 10 μM. The

O_2 level was saturating for all these assays, but was maintained below 55 μM, as the affect of high O_2 levels on the three separate (hydrogenase) enzymes is not known. The double reciprocal plot yields a line equation, for which kinetic parameters can be calculated. The Km from this data is referred to herein as the half saturation affinity for H_2, because in our case the kinetic constants are for a whole cell system rather than for the pure enzyme (the latter being the conventional system for such determination).

Please replace Table 1 on page 23, with the following amended Table 1.

Table 1. Variations of Growth Conditions for Obtaining Respiratory H2 Oxidizing Activity

Condition No.	Medium	Description Gas atmosphere	Activity (Nmoles H2/min/109 cells)
1	Blood Agar	Anaerobic Mix	11.9 ± 1.5
2	Blood Agar	Anaerobic Mix, but 2% O_2	3.2 ± 0.4
3	Blood Agar	~~Campypak~~CAMPYPAK system	2.1 ± 0.3
4	Blood Agar	Anaerobic Mix, but 8% O_2	<0.2
5	Blood Agar	Anaerobic Mix, but without H2	7.2 ± 1.2
6	Luria Bertani Agar	Anaerobic Mix	2.8 ± 0.4
7	Luria Bertani Agar	Anaerobic Mix, but 2% O_2	<0.2
8	Luria Bertani Agar	~~Campypak~~CAMPYPAK system	1.2 ± 0.1
9	Luria Bertani Agar	Anaerobic Mix, but 8% O_2	<0.2
10	Luria Bertani	Anaerobic Mix, but no H_2	1.3 ± 0.3

Please replace the paragraph beginning at page 23, line 8, with the following amended paragraph.

* Anaerobic mix consists of 10% H_2, 5% CO_2, balance N_2. After sparging with this mixture, O_2 levels were below 0.2% partial pressure, but were not anaerobic. Results are mean ±

std. Dev. For 5 replicate independent samples. BA = Blood Agar LB = Luria Broth ~~Campypak~~ CAMPYPAK is a H_2 and CO_2 generating system that depletes O_2; initially the atmosphere is air, but less than atmospheric O_2 is achieved.

Please replace the paragraph beginning at page 31, line 30, with the following amended paragraph.

Construction of *H. hepaticus hyaB* (HH 0057) and mutant strain by insertional mutagenesis. The hyaB gene which encodes for the large subunit of hydrogenase lies between hyaA (which encodes for the small subunit of hydrogenase) and hyaC (which encodes for a cytochrome oxidase, part of the hydrogenase operon) (Figure 4). The downstream gene hyaD is also a part of the hydrogenase operon since it is an orthologue of the hydD gene of H. Pylori shown to be required for synthesis of hydrogenase. Primers hyaBF (5'ttcgtggtatgaggataatcagcc-3'; SEQ ID NO: 18) and hyaBR (5'aataaagcacaactcccgtgagag-3'; SEQ ID NO: 19) were used to PCR amplify a 1303-bp fragment using wild type H. Hepaticus ATCC 51449 genomic DNA as template. This fragment contained partial sequences of hyaB and its adjacent gene, hyaC. The PCR fragment was ligated into pGEM-T vector (~~Promega~~PROMEGA) following the manufacturer's instructions. The cloned construct was obtained by transforming the ligation mixture into E.coli DH5α. Subsequently, a chloramphenicol (Cm) resistance cassette was inserted into a unique AvaI site within hyaB giving the construct, pGEM-T:hyaB:Cm. The recombinant plasmid was introduced into H. Hepaticus by electrotransformation (pulse of 2.5kV in a transporator plus apparatus, BTX). As a result of allelic exchange, the hyaB mutant strain was obtained.

Please replace the paragraph beginning at page 33, line 18, with the following amended paragraph.

^{14}C labeled amino acid uptake ability of *hyaB* mutant and wild type. In order to investigate whether membrane-bound hydrogenase can use hydrogen as an energy substrate for uptake of amino acids, *H. hepaticus* wild type and hyaB mutant cells were grown overnight on a shaker at 37°C in MHB with 2% FBS in 160 ml serum bottles, four bottles per strain. These

tightly sealed bottles were previously sparged with N2 to expel all the air and injected with 2% O2 and 5% CO2 (v/v of head space). After the OD600 reached 0.08, 10%H2 (v/v of head space) was injected into two of the bottles for each strain and 10% argon (v/v of head space) into the other two. After 2 hrs, ^{14}C uniformly-labeled amino acids (Amersham Biosciences AMERSHAM BIOSCIENCES CFB104, specific activity in the medium within the serum bottles was 1.0 μCi/ml. For the 0 hr time reading, 2 ml of the culture suspension was immediately withdrawn from the bottles (after injecting the label and mixing solution) using a sterile syringe, and four separate samples (2 ml) were added to wells of a filtering manifold unver vacuum. The filter used was 0.22 μm membrane (~~Millipore~~ MILLIPORE, GSWP 02500). After filtering the cell suspension, the membrane was washed with two separate l ml aliquots of sterile phosphate buffered saline (PBS) pH 7.2 to wash off the ^{14}C amino acids externally adhering to the cells, and then the membranes were transferred into individual scintillation vials. Five ml of the fluor (~~ScintiVerse~~SCINTIVERSE, Fischer Scientific) was added. Scintillation spectrometry was done as described previously (Maier et al., (1988) J Bacteriol 170: 1986-1989). The background level uptake of ^{14}C amino acids (at 0 hr) was estimated after averaging the four separate 2 ml samples. After background sampling, the bottles were allowed to shake at 37°C for 2 and 5 hr, and 2 ml samples (total of 8 replicate samples) of the culture suspension were taken and filtered as described above. The average cpm/2 ml of filtered cells at 0 hr was subtracted from the averaged 2 and 5 hr time points, resulting in the data shown in Table 4.

Please replace the paragraph beginning at page 35, line 22, with the following amended paragraph.

Mice were euthanized 21 weeks post inoculation and the liver and cecum were excised. Liver (left, right, caudate and part of the median lobe) and cecum (entire tissue after taking out sample for histopathology), were first homogenized using a tissue tearor (~~Biospec~~BIOSPEC Products, Inc., Bartlesville, OK). The homogenized tissue suspensions (200 μl) were plated on BA plates with triple antibiotic to check for the presence or absence of H. Hepaticus. Growth, if present, was confirmed by microscopy, catalase test and by PCR. Part of the median lobe of the liver was used for hisotpathology.

Please replace the paragraph beginning at page 35, line 29, with the following amended paragraph.

Quantitative analysis of liver and cecum. It is commonly acknowledged that counting colonies of H. Hepaticus is a challenging protocol, as the colonies tend to stick to one another, and to spread out over the plate. Hence, we performed a real time PCR to quantify *H. hepaticus* in the tissues. In addition, real time PCR is a rapid and sensitive technique which has been used to amplify and detect *H. hepaticus* DNA from tissue isolates of infected mice. Following homogenization of the liver and cecum, the DNA was extracted from 25 mg of the tissue using the Dneasy tissue kit (~~Qiagen~~QIAGEN). Quantitative analysis was performed by real time PCR (iCycler Thermal cycler, BIO-RAD) using H. Hepaticus specific cdtB primers (cdtBF 5'-GGCTAGATACAAGAATCGCTAAT-3'; SEQ ID NO: 20 and cdtBR 5'-CTACCTACTACCGCATAATCAAG-3'; SEQ ID NO: 21) which produce a 109 bp amplicon. Specific primers and probe were designed using the Beacon Designer Software (version 3.0, BIO-RAD). Primers and probe for the *cdtB* gene (encoding for subunit B of the cytolethal distending toxin) were shown to be highly specific for *H. hepaticus* by doing a BLAST against known genomes.

Please replace the paragraph beginning at page 36, line 11, with the following amended paragraph.

Real time PCR analysis was performed by using a 50 µl mixture containing 25 µl of iO ~~Supermix~~ SUPERMIX (BIO-RAD), 200 nM each of cdtF, cdtR primers, cdtB probe and 10 µl of tissue DNA (equivalent to 1.25 mg tissue). Conditions for real time were 1 cycle at 95° C for 3 min, 40 cycles each of 95° C for 30 s and 58° C for 30 s. Samples ranging from 102 to 107 femtograms of *H. hepaticus* genomic DNA was used to generate a curve. From the standard curve of threshold cycle v/s log starting quantity in femtograms, the amount of DNA in the unknown tissue samples was estimated and converted to the number of copies of *H. hepaticus* genome using the following calculations: